



***In vitro* antibacterial activity of endophytic fungus *Aspergillus flavus* IBRL-C8 from *Senna siamea* against Gram-bacteria**

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ABSTRACT

Aims: To evaluate the antibacterial efficacy of ethyl acetate extract of *Aspergillus flavus* IBRL-C8 against Gram-positive and Gram-negative bacteria.

Methodology and results: In this experiment, an endophytic fungus which identified as *A. flavus* IBRL-C8 was extracted using ethyl acetate and methanol, from *Senna siamea*, prior to *in vitro* antibacterial test on eight Gram-bacteria. The results were significantly more enunciated to the ethyl acetate extract since the Gram-bacteria signified 9.0 to 20.0 mm of inhibition zones on Muller Hinton Agar (MHA) during disc diffusion assay. Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of the extract were ranged from 125-1000 µg/mL and 125-2000 µg/mL, respectively. Time-kill assay depicted the ethyl acetate extract of *A. flavus* IBRL-C8 exceptionally retarded methicillin-resistant *Staphylococcus aureus* (MRSA) and also manifested extended antibacterial activity. The maximum reduction in cell numbers occurred at 2MIC concentration (250 µg/mL) during the interval time of 16 h. The malformations noticed from microscopic observations where the transformation of structural annihilation from regular spherical morphology to non-spherical shape with an irregular surface and also disruption around the cell membrane when the MRSA treated with ethyl acetate extract of *A. flavus* IBRL-C8.

Conclusion, significance and impact of study: This study proposed the ethyl acetate extract of *A. flavus* IBRL-C8 as a potential antibacterial agent against MRSA infection, which can be useful in pharmaceutical application.

Keywords: *Aspergillus flavus*, ethyl acetate extract, methanolic extract, antibacterial activity, Gram-bacteria

INTRODUCTION

Recently, the frequency of health problems caused by cancer, drug-resistant bacteria, parasitic protozoans, and fungi are continuously increasing. It is known that the usage of uncontrolled synthetic drugs can lead to the development of multiple drug-resistant microbes. This condition is getting worse as some pathogenic bacteria show resistance to virtually all available drugs (Lister, 2006). Therefore, rigorous search for a novel antibacterial agent is needed to provide more options in ameliorating the human diseases. Natural resources such as endophytes provide several valuable compounds with medicinal values that are significant to be explored.

Endophytes are microbes including bacteria and fungi that reside in living tissues of a plant without triggering any adverse effect on the host (Bacon and White, 2000).

The symbiotic mutualism relationship allows the endophytes to obtain nutrients from the host plant. In return, the microbes produce certain functional metabolites that can enhance the viability of the host plant and protect it from pathogenic organisms (Tan and Zou, 2001). There are several recognised bioactive compounds derived from the fungal endophytes, specifically alkaloids, terpenoids, quinines, isocoumarin derivatives, flavonoids, phenols, peptides and phenolic acids (Gangwar *et al.*, 2015). Mirroring to these compounds, the endophytic fungi have potential as a compatible agent for antibiotic, immunosuppressant, anti-cancer, antibacterial, antifungal (Chandra, 2012), antiviral, antioxidant, insecticide and anti-diabetic (Demain, 1999). Thus, endophytes are undoubtedly a promising source for novel antibacterial metabolites. Secondary metabolites from endophytes are imperative alternatives to surmount

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the increasing level of drug resistance by human pathogens since there are inadequate numbers of effective antibiotics against diverse bacterial species (Song, 2008; Yu *et al.*, 2010).

Senna siamea leaf belongs to the family Fabaceae or Caesalpinaceae (Doughari and Okafor, 2008). This species is known as one of the medicinal plants among local folks especially in treating diabetes, insomnia, hypertension, asthma, constipation and diarrhoea (Mohammed *et al.*, 2012). Mun'im *et al.* (2013) have proven a symbiotic relationship between endophytic fungi and *S. siamea* as a host plant. They have isolated five endophytic fungi colonies that possessed α -glucosidase enzyme, an anti-diabetic agent. Therefore, in this recent communication, we report on the antibacterial activity of ethyl acetate extract of *Aspergillus flavus* IBRL-C8, with highlights on disc diffusion assay, antibacterial susceptibility test, time-kill curve and also an investigation on its effect on cells alteration.

MATERIALS AND METHODS

Chemicals and endophytic fungus

All chemicals were purchased from Sigma-Aldrich (USA), Hi-Media Lab. LTD (India) and Fluka (USA), unless stated otherwise. An endophytic fungus isolated from *S. siamea* leaves and later identified as *A. flavus* IBRL-C8 was applied throughout this experiment (Nurhaida, 2019). *S. siamea* leaves was picked from *S. siamea* tree, which was grown near Universitas Syiah Kuala, Kopelma Darussalam (5°34'40"N), Banda Aceh, Indonesia.

Cultivation of endophytic fungus

Yeast extract sucrose (YES) medium was supplemented with the aqueous extract of *S. siamea* leaves. Then, two plugs of endophytic fungus (7 days old) were cultivated in 250 mL Erlenmeyer flask containing 100 mL of the YES medium (Tong *et al.*, 2011). The final pH of the medium was set to 5.8. All samples in the flasks were agitated using an orbital shaker at a speed of 120 rpm, 30 °C. After 20 days of fermentation process, the broth which containing the fungal mycelia was filtered using a muslin cloth and filter paper (Whatman filter paper No. 1). Next, the fungal mycelia was subjected to freeze-dryer (Labconco, USA), prior to extraction step.

Solvent extract of *A. flavus* IBRL C-8

Method of Tong *et al.* (2014) was applied at this section. There were two parts of extraction processes specifically the extra- and intracellular compounds. The extracellular compound was extracted by using ethyl acetate. The solvent and fermentation broth was mixed with a volume ratio of 1:1 in a separating funnel. After that, the solvent-broth combination was slowly shaken for 1-2 min and then was kept stagnant until two clear immiscible layers formed. The upper layer of the mixture was collected and evaporated into dry biomass under a reduced pressure

using a rotary evaporator (EYELA, Japan). As for intracellular compound, the freeze dried fungal mycelia obtained from the aforementioned step was immersed in methanol (1:50; w/v) for 24 h, before filtration with Whatman filter paper No. 1. Finally, the solvent part was evaporated via a rotary evaporator and kept in a fume hood until the extract paste produced.

Determination of antibacterial activity of fungal crude extracts

Test microorganisms

Eight human pathogenic microorganisms *viz.* 4 Gram-positive bacteria (MRSA, *Staphylococcus aureus* ATCC 12600, *Bacillus cereus* ATCC 10876 and *Bacillus subtilis* IBRL A3) and 4 Gram-negative bacteria (*Escherichia coli* IBRL 0157, *Klebsiella pneumonia* ATCC 13883, *Shigella boydii* ATCC 9207 and *Salmonella typhimurium*) were selected for this study. All of the tested microorganisms were supplied by Industrial Biotechnology Research Laboratory (IBRL), School of Biological Sciences, Universiti Sains Malaysia.

Disc diffusion assay

Preliminary screening was performed according to a disc diffusion assay (Clinical and Laboratory Standards Institute, 2006). The bacteria were streaked on Muller Hinton agar (MHA) plates by a cotton swab. Next, the extract paste was diluted in methanol to generate a concentration of 1 mg/mL. Twenty microlitre of the extract was pipetted on sterile discs paper (6 mm) which were placed on the surface of inoculated MHA. The plates were incubated at 37 °C for a day. The appearance of a clear inhibition zone on the plates signified the antibacterial capacity of the tested extract. Chloramphenicol and methanol were applied as positive and negative control, respectively.

Antibacterial susceptibility test

The minimal inhibition concentration (MIC) of the fungal extract was measured through a colourimetric broth microdilution method (Jorgensen and Ferraro, 2009). The analysis was performed in sterile 96 wells microtiter plates (Tong *et al.*, 2014). Sterile Mueller-Hinton broth (MHB) containing 2 mg/mL of the crude extract was prepared as a stock. The stock underwent a serial dilution using two-fold strength sterile broth medium. The final concentrations of the extract in each well were ranged from 62.5-2000 μ g/mL. Then, 100 μ L of extract was added into 100 μ L bacterial suspension. The negative control and sterility of the medium were also provided. The bacterial growth was developed at 37 °C for 24 h. Forty microlitre of P-iodonitrotetrazolium violet salt or INT, at a concentration of 0.2 mg/mL, was added to the plates. The INT discolouration, from yellow to purple, indicated the bacterial growth. The minimal bactericidal concentration (MBC) test was executed by using a

streaking technique. Any wells from the MIC test that showed no microbial growth were streaked on the nutrient agar (NA). After that, the plates were incubated at 37 °C for a day.

Time-kill assay

The test was performed as reported by Yadav *et al.* (2015). Ethyl acetate extract of *A. flavus* IBRL-C8 was prepared at a concentration of ½MIC, MIC and 2MIC (62.5, 125 and 250 µg/mL, respectively) in different tubes, before adding the bacterial suspension. A single tube containing only bacterial suspension was assigned as a control. All tubes were incubated at 37 °C for 48 h. For the plate count method, 100 µL of the sample was pipetted out and spread on NA plates at the interval time of 0, 2, 4, 6, 8 and 48 h.

Methicillin-resistant *Staphylococcus aureus* cells alteration

Fixation was done on the ethyl acetate extract of *A. flavus* IBRL-C8 treated and untreated MRSA cells, prior to morphological and structural observation through scanning electron microscopic (SEM) and transmission electron microscopic (TEM). For the treated cells, 1 mL of the MRSA cell suspension (concentration of 1×10⁸ CFU/mL) and 1 mL of ethyl acetate extract (MIC=125 µg/mL) were mixed in Mueller Hinton broth. The flask was agitated in a rotary shaker at 150 rpm, 37 °C. After 24 h, the mixture sample was centrifuged at 1500 × g for 10 min and fixed with McDowell-Trump fixative. Method of Mascorro and Bozzola (2007) was applied for the next processes. All samples were observed under SEM (FESEM Carl Zeiss Leo Supra 50 VP, Germany) and TEM (Phillips CM12, Netherlands) (Mascorro and Bozzola, 2007).

Statistical analysis

SPSS version 20 (SPSS, Chicago, USA) was employed to analyse the triplicate data resulted from the experiment. Statistical significance was considered accepted at $p < 0.05$.

RESULTS AND DISCUSSION

Table 1 demonstrates the antibacterial activity of *A. flavus* IBRL C-8 on disc diffusion assay. As observed, the activity exhibited by the ethyl acetate extract was more significant than methanolic extract ($p < 0.05$). The diameter of inhibition zones of ethyl acetate extract ranged from 9 mm to 20 mm, of which the maximum diameter was displayed by Gram-positive bacteria, MRSA. Plus, the inhibitory efficacy of ethyl acetate extract on MRSA was relative to chloramphenicol (Kundan *et al.*, 2018; Mohd *et al.*, 2020). Contrarily, the methanolic extract only retarded MRSA and *B. subtilis* with a diameter of 10.7 ± 0.9 mm and 10.3 ± 0.5 mm, respectively.

Table 1: Antibacterial activity of ethyl acetate extract of endophytic fungus *A. flavus* IBRL-C8 on Gram-positive bacteria and Gram-negative bacteria.

	Test bacteria	Ethyl acetate extract	Methanol extract	Positive control	Negative control
Gram positive	MRSA	+++	+	+++	-
	<i>S. aureus</i>	++	-	+++	-
	<i>B. cereus</i>	++	+	+++	-
	<i>B. subtilis</i>	++	-	+++	-
Gram negative	<i>E. coli</i>	++	-	+++	-
	<i>K. pneumoniae</i>	+	-	+++	-
	<i>S. bodyii</i>	++	-	+++	-
	<i>S. typhimurium</i>	++	-	+++	-

+++ : ≥ 20 mm, ++ : 12–19 mm, + : ≤ 11 mm, - : no inhibition zone observed.

Table 2: The MIC and MBC values for the ethyl acetate extract against all tested bacteria.

Pathogenic bacteria	MIC (µg/mL)	MBC (µg/mL)	Ratio of MBC:MIC
Gram positive			
MRSA	125	125	1 (Bactericidal)
<i>S. aureus</i>	125	125	1 (Bactericidal)
<i>B. cereus</i>	125	125	1 (Bactericidal)
<i>B. subtilis</i>	125	125	1 (Bactericidal)
Gram negative			
<i>E. coli</i>	125	250	2 (Bactericidal)
<i>K. pneumoniae</i>	1000	2000	2 (Bactericidal)
<i>S. bodyii</i>	1000	1000	1 (Bactericidal)
<i>S. typhimurium</i>	1000	2000	2 (Bactericidal)

The ethyl acetate extract signified an exceptional result since it depicted notable antibacterial activity against all Gram bacteria, particularly the Gram-positive bacteria. The Gram-negative bacteria are equipped with double-layer membranes built from peptidoglycan and lipopolysaccharide (Silhavy *et al.*, 2010). These structures provided the bacteria with a restricted diffusion towards any bioactive compounds (Burt, 2004). Therefore, the Gram-positive bacteria were more susceptible to the ethyl acetate extract than the Gram-negative bacteria. Besides that, the ethyl acetate solvent owns a capability to withdraw the extracellular secondary metabolites from endophytic fungi (Garcia *et al.*, 2012). Conversely, the methanol solvent can extract out the intracellular compound. This statement agreed with Musavi and Balakrishnan (2014).

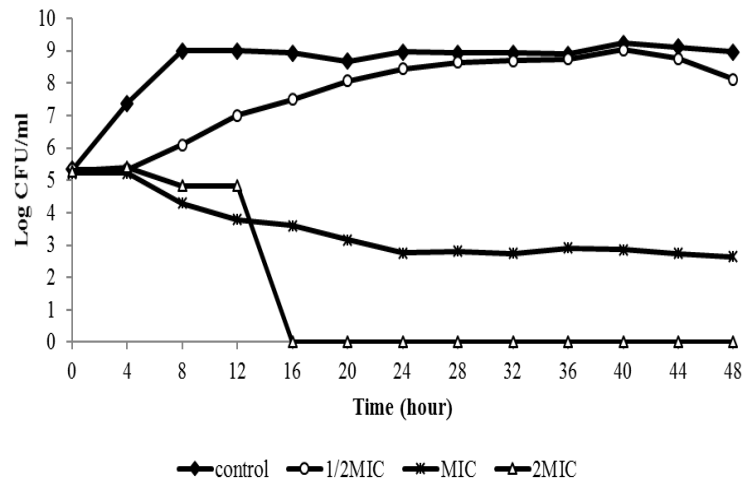


Figure 1: Time-kill curve of ethyl acetate extract of *A. flavus* IBRL-C8 against Methicillin-resistant *S. aureus* (MRSA) at different extract concentrations.

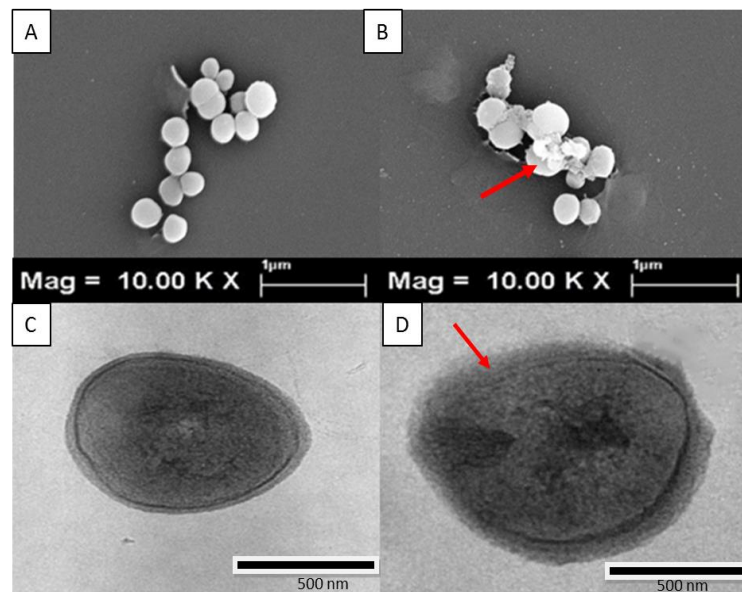


Figure 2: SEM and TEM micrographs of MRSA treated with 250 µg/mL of *A. flavus* IBRL-C8 ethyl acetate extract after 24 h exposure. (A) MRSA cells without extract treatment under SEM observation, (B) MRSA cells with extract treatment under SEM observation, (C) MRSA cells without extract treatment under TEM observation and (D) MRSA cells with extract treatment under TEM observation.

Aspergillus flavus has a few decades of history with an antibiotic production which is known as aspergillilic acid. It is also related to the production of other antimicrobial properties including linoleic acid (Dilika *et al.*, 2000) and kojic acid (Wu *et al.*, 2018). These chemicals, by and large, are reported to inhibit *B. cereus*, *B. pumilus*, *B. subtilis*, *Micrococcus kristinae*, *S. aureus*, *Listeria monocytogenes*, *E. coli*, and *S. typhimurium*. Eliwa *et al.* (2017) have isolated N-phenylbenzamide derivative from

A. flavus, specifically novel 5-chloro-2-methoxy-N-phenylbenzamide that might also serve as an antimicrobial agent.

MIC value is essential in determining an accurate dose for growth inhibition of specific microorganism, especially in treatment of the diseases in human (Andrews, 2001; Nor Afifah *et al.*, 2010). The antibacterial susceptibility test outcomes for ethyl acetate extract, prepared from *A. flavus* C-8, are presented in Table 2. Both MICs and

MBCs for Gram-positive bacteria were accurately at a concentration of 125 µg/mL. In contrast, the MICs and MBCs for Gram-negative bacteria were at a higher range i.e. 125-1000 µg/mL and 250-2000 µg/mL, respectively. As recorded, the MBCs for Gram-negative bacteria were significantly ($p < 0.05$) superior than the Gram-positive values. In this experiment, the results suggested the Gram-positive bacteria were more susceptible to the ethyl acetate extract of *A. flavus* IBRL-C8 than the Gram-negative bacteria. MBC is defined as the lowest concentration that exhibited no visible microbial growth. Meanwhile, the ratio of MBC:MIC evaluates the bacteriostatic and bactericidal activity.

The broad range of MICs represented the different susceptibility levels of the Gram-bacteria to the extract. It usually happens when the microorganisms are exposed to certain bioactive chemicals in the extract. This event commonly follows by low MIC (Ciusa *et al.*, 2012). The concentration-dependent occasion was experienced by the Gram-negative bacteria due to higher MBCs than MICs. Thus, higher concentration of the target extract or compound is required to eradicate the bacteria (Tong *et al.*, 2017). By evaluating the ratio of MBC:MIC, our current findings have proven the "cidal" effect of ethyl acetate extract of *A. flavus* IBRL-C8 on Gram-bacteria. The extracts are considered bearing the bactericidal effect if the ratio of MBC:MIC is four or less than four. Commonly, the bactericidal activity depends on the period of drug exposure at MIC or 2MIC (Levison and Levison, 2009). Based on the disc diffusion assay, MICs, MBCs and also the bactericidal effect of *A. flavus* IBRL-C8 extract, we decided to choose MRSA for the next experimental step.

Figure 1 illustrates the time-kill curve of *A. flavus* IBRL-C8 extract against MRSA. Overall, the growth of MRSA at MIC concentration (125 µg/mL) and 2MIC concentration (250 µg/mL) displayed bacteriostatic and bactericidal activities, respectively. The graph lines showed the increase of extract concentration triggered a reduction in the microbial growth. It revealed a concentration-dependent kill curve for MRSA. The bactericidal event can be observed by a gradient drop in the cell numbers from the interval time of 0 until 12 h. After that, it was tailed with a drastic shortfall at the interval time of 16 h. At this moment, the MRSA cells did not show any sign of growth, the possibility of cells were killed, indicated by a straight baseline in the graph. It can be concluded that the ethyl acetate extract from *A. flavus* IBRL-C8 possessed a bactericidal effect at a higher concentration and bacteriostatic at a lower concentration. Most of the ethyl acetate extract in low concentration indicates bacteriostatic effect against bacteria (Geidam *et al.*, 2015; Taufiq and Darah, 2018). Furthermore, there was no post-antibiotic effects (PAE) monitored in all concentrations tested.

Figure 2 demonstrates the result for MRSA cells alteration under SEM and TEM observation. Under SEM view, the untreated MRSA cells have coccal shape and smooth cell surface (Figure 2A), whereas the cells exposed in ethyl acetate extract revealed non-spherical shape with an irregular surface and formed cavities.

Lysed and dead debris was also found around the cells (Figure 2B). As observed under TEM, the coccal shape of the untreated structure was perceptibly observed. It has dense cytoplasm and homogenous cell membrane with approximately 25-30 nm thickness (Figure 2C). Conversely, the treated extract showed a notable cell membrane disruption as indicated by the arrow in Figure 2D. There was no distinct symmetrical bilayered cell membrane appeared and most probably due to the nucleoid damage. The severe damages of cell membrane have led to MRSA cell death. It is worth to mention that a similar phenomenon was observed during MRSA exposure to 10 µg Zymar (gatifloxacin plus benzalkonium chlorine). The cell wall surface of the bacteria was altered and experienced greater pleomorphism (Monson *et al.*, 2010).

CONCLUSION

The susceptibility tests have recognised the endophytic fungus *A. flavus* IBRL-C8 as a promising antibacterial agent against Gram-bacteria, especially towards MRSA. The alterations of MRSA cells to the non-spherical, irregular cell surface, cavities and even membrane disruption have proven the capability of the crude extract in demolishing the MRSA. To the best of knowledge, the ethyl acetate extract of *A. flavus* IBRL-C8 isolated from *S. siamea* leaves has never been evaluated for anti-MRSA activity before. Therefore, based on the present study, further purification of bioactive compound(s) from this endophytic fungus including the physicochemical characterisation and also *in vivo* study are suggested.

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